Chapter 4

Bitter Sweetness of Malignant Melanoma: Deciphering the Role of Cell Surface Glycosylation in Tumour Progression and Metastasis

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Additional information is available at the end of the chapter

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Abstract

Malignant melanoma is the sixth most commonly diagnosed cancer in developed countries. Like in many cancers, survival rates are closely associated with the time of melanoma detection. Regrettably, most cases of melanoma are caught at diffuse state and methods used in clinical practice and experimental trials are not effective. Thus, there is a great interest in discovering biomarkers that could be used for screening those with melanoma, as prognostic and prediction factors as well as new potential targets for melanoma treatment. For this purpose, many groups have examined alteration in the structure and expression of carbohydrate part of glycoconjugates to identify changes that occur with melanoma. The observed changes include increased β1,6 branching correlating with higher abundance of polylactosamine extension, increased sialylation accompanied by differences in the position of sialic acid residues, increased fucosylation, higher levels of T and Tn antigens as well as changes in the expression of ganglioside structures. As a consequence of glycan modification, the loosened matrix adhesion, increased motility, higher invasive potential and metastasis formation have been observed. Growth and migration of melanoma cells have been also found to be stimulated by advanced glycation end products. Biomarker discovery is a multi-step process and the recent glycomic data on melanoma are mostly related to the discovery phase, as the first one leading to validation and standardisation steps.

Keywords: glycosylation, glycation, malignant melanoma, tumour-associated carbohydrate antigens

1. Introduction

The population of patients with cutaneous melanoma is one of the most rapidly increasing cancer groups worldwide over the last 50 years in most fair-skinned populations [1, 2]. The vast majority of cases (almost 85%) occur in developed countries, where melanoma is the sixth most commonly diagnosed cancer. Despite the improvements in the diagnosis thereof, the best chance of melanoma recovery remains the surgical removal of a thin early-stage lesion, because methods used on large scale in clinical practices as well as experimental trials are not able to cure this type of cancer at diffuse state [3, 4]. Palliative treatment for inoperable recurrence or metastatic disease is frequently toxic and ineffective. Thus, appropriate means for predicting prognosis or effective treatments are still needed.

Understanding the biology of tumour cells is an important factor for the development of new strategies for cancer treatment. Unfortunately, reliable biomarkers are not available for the vast majority of cancers. Macro-molecules present at the cell membrane surface and in the membranes themselves constitute an important field for study in the understanding of cancer cell behaviour. Nowadays, investigation on structural properties and function of cancer-associated glycosylation changes, as indicators of tumourigenesis, is gaining more attention in order to discover new markers suitable for early detection, for differentiating between benign and malignant stages and for therapeutic purposes. For a long time carbohydrates have been merely regarded as an integral structural component of glycoconjugates (glycoproteins, glycolipids and proteoglycans) and a storage material. Although they are ubiquitous constituents of almost all living organisms, glycoconjugates were believed to be deprived of any biological function. Progress in glycoconjugates research due to application of new powerful tools has enabled researches to discover the broad range of biological activities in which carbohydrates are involved [5–8]. Glycoconjugates participate in several processes, including protein conformational stability, protection from proteolytic degradation, protein thermal stability, biological activity, protein targeting, circulating life-time and their ultimate fate, immunogenicity, the transduction of information between cells, sperm-egg interactions, leukocyte traffic to sites of inflammation, leukocyte migration (homing) to lymphoid organs, blood clotting, apoptosis and host-pathogen interactions. Moreover, changes in glycoconjugates have been proved to be associated with a number of pathological processes, for example, carbohydrate deficiency diseases, inflammation, allergy, rheumatoid arthritis, thrombosis, infarction, diabetes and cancer [7, 9–12]. The aim of this chapter is to highlight the contribution of the defined tumour-associated carbohydrate antigens present on the cell surface of melanoma cells to their behaviour during tumour progression and metastasis, as well as to present glycomic opportunities in defining markers for melanoma early detection, disease progression or predicting therapy outcome that might help to defeat one of the deadliest forms of cancer.

2. Glycoconjugates and cancer

Glycosylation is the most frequent post-translational modification of macro-molecules. Carbohydrate part (glycan) biosynthesis involves various types of glycosyltransferases,

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glycosidases and sugar nucleotides [8, 13]. As glycosylation is not template-driven, but is indirectly controlled by a number of genes (1–2% of translated genome) and the environmental factors integrate at the level of glycan biosynthesis, the relative amounts and structure of glycans is cell-, tissue- and species-dependent [14]. The biosynthetic basis of such diversity consists in the alteration in the activity of various glycosyltransferases and competition between enzymes for acceptor intermediates during glycan elongation. Additionally, in cancer cells the activity of glycosyltransferases and glycosidases is controlled by other factors such as the levels of nucleotide sugars and their transporters, the expression of chaperons that regulate protein folding and quality control of proteins, endogenous lectin as well as by altered expression of the enzymes engaged in biosynthetic process together with their proper localisation [15]. Recently, it has been shown that the aberrant expression of glyco-genes in cancer is also due to aberrant promotor methylation. In melanoma a difference in methylation of 20 genes involved in O-glycosylation has been stated [16]. These results suggest new potential targets for melanoma treatment, and indicate that the methylation status of selected glyco-genes might be used for prognostic purposes.

The hallmarks of all types of human, as well as experimental rodent cancers include profound changes in the structure and expression of carbohydrate part of glycoconjugates, resulting from activation of particular oncogenes or rearrangements of glycan biosynthetic pathways [1, 11, 12, 17]. Generally, cancer-associated changes in glycosylation profile are associated either with the expression and secretion of inappropriate glycosylated molecules or the appearance of new antigens (onco-foetal or *de novo* synthesised antigens). Some of the cancer-associated carbohydrate antigens have found their clinical application as a target for the diagnosis of different types of tumours (breast, ovarian and prostate cancers) or as therapeutic agents (glycoconjugate vaccines) [7, 9, 11, 12, 18–26]. Interestingly, among at least 100 cancer biomarkers used currently for the diagnosis of different types of tumours, the vast majority includes glycoproteins and glycolipids, and they are measured immunochemically using monoclonal antibodies [27]. However, these monoclonal antibodies against glycoprotein are in most cases aimed not towards the glycan epitope, but towards the protein chain. The most frequently observed changes in glycosylation structure during malignant transformation are the extensive expression of β1,6-branched N-glycans, the increased expression of bisected N-glycans, increased cell surface sialylation frequently accompanied by differences in the position of sialic acid residues including the expression of onco-foetal α-2,8-linked polysialic acid, the expression of core fucosylated and non-fucosylated paucimannose-type structures, premature termination of O-glycan biosynthesis in mucins leading to the presence of the so-called pan-tumour antigens, i.e. T (Galβ1–3GalNAc-α1-O-Ser/Thr), Tn (GalNAc-α1-O-Ser/Thr) and sialyl-Tn (Siaα2-6GalNAc-α1-O-Ser/Thr) mucin antigens, abnormalities in the expression of ABO blood group and tissue antigens [15].

Protein-carbohydrate interactions have not only biological but also medical implication, since glycosylation profile is dynamically modified by many intra- and/or extra-cellular stimuli. Additionally, these interactions are involved in the control of cell homeostasis and its social behaviour. Therefore, alterations in carbohydrate structures of glycoconjugates including cell adhesion molecules, commonly found in various tumours, are considered to be the basis for

abnormal social behaviour of tumour cells, such as invasion to the surrounding tissue and metastasis, loose of cell-cell contact and epithelial-mesenchymal transition (EMT) [15, 18, 28–34]. These macro-molecules could also significantly change antigenicity and immunogenicity of tumour cells and therefore promote tumour progression by chronic inflammation and angiogenesis [24].

3. Glycosylation and melanoma

Over 5000 cell lines are currently available for studying cutaneous and ocular melanoma, which covers different stages of the disease progression from primary melanomas to metastases in distinct organs [35]. The most frequently used model to study the linkage between glycosylation and metastatic behaviour of melanoma cells is B16 murine melanoma cell line and its sub-lines of a different metastatic potential. Detailed analysis of B16 sub-lines with high- and low-metastatic potentials has revealed that although these sub-lines expressed comparable amount of sialic acids, α2,3-linked sialic acids were predominantly found in high-metastatic sub-line, while α2,6-linked sialic acids were observed in low-metastatic sub-line [36]. Other studies performed on a poorly metastasising wheat germ agglutinin-resistant mutants of B16 melanoma cells have proved that the variant cell line displayed well-defined changes in its cell surface glycosylation profile in comparison to wild-type cells, involving the decrease in the number of side chains in oligosaccharides, the loss of sialic acids α2,3-linked to galactose, concomitant with the increase in the amount of fucose α1,3-linked to N-acetylglucosamine [37]. Such cells were less adherent to extracellular matrix components and showed decreased metastatic potential. The observed effects resulted from a 60-fold increase in α1,3-fucosyltransferase activity, while sialyltransferase activity did not decrease significantly [38]. Participation of sialic acids in metastasis formation has been also demonstrated by transfection of murine B16, JB/RH and JB/MS cells with gene for α1,3-galactosyltransferase (α1,3GT). α1,3GT competes with α2,3-sialyltransferase and α2,6-sialyltransferase for the same acceptor, i.e. N-acetyllactosamine moieties (**Figure 1**) [39]. The transfected cells showed reduced metastasis formation which was caused by the reduction of cell membrane sialylation. Similar great reduction of metastatic capacity has been observed after the use of swainsonine (SW), a competitive inhibitor of Golgi α-mannosidase II which stops N-oligosaccharide synthesis on hybrid and high-mannose-type structures preventing the synthesis of complex-type structures (**Figure 1**) [40]. It has also been demonstrated that the loss of sialylated lactosamine antenae and decreased branching of N-oligosaccharides on B16-F10 melanoma cells (cells of high incidence of lung colonisation) reduced their pulmonary colonisation when the cells were injected into the circulation of syngeneic mice [41]. This was consistent with the observation that SW treatment of athymic nude mice bearing human MeWo cells significantly reduced solid tumour growth and inhibited tumour cell proliferation both *in vitro* and *in vivo* [42]. Interestingly, SW has been reported to show evidence of clinical efficacy in a phase I clinical trial [43]. Metastatic capacities of highly metastatic B16-hm melanoma cells have also been down-regulated by introduction of β1,4-N-acetylglucosaminyltransferase III (GnT-III) gene, which codes the enzyme that catalyses the formation of bisecting N-acetylglucosamine

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in N-oligosaccharide chains [44]. One of the targets of GnT-III in the transfected cells was E-cadherin. GnT-III gene transfected cells showed increased E-cadherin-dependent cell-cell adhesion and suppression of lung metastasis formation as well as decreased level of cell adhesion to laminin and collagen [44, 45]. Taken together, these results strongly suggested that highly branched and sialylated N-oligosaccharides present on cell surface glycoconjugates contribute to effective melanoma cell metastasis. Interestingly, N-glycosylation in human melanoma SK-MEL-2 cells has also been found to play an important role in maintenance of viability thereof through the regulation of insulin-like growth factor-1 receptor translocation to the cell surface [46, 47].

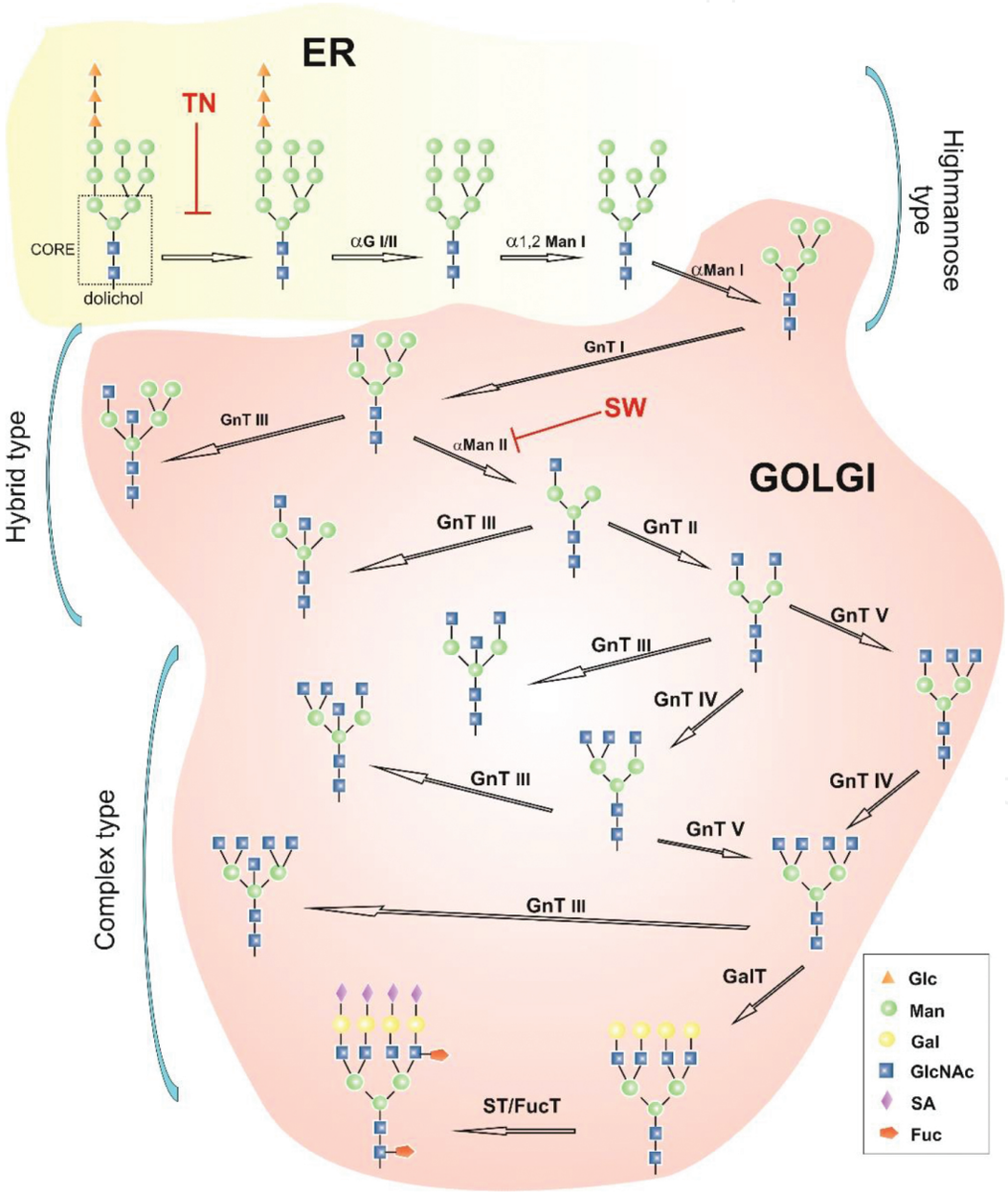

Figure 1. Structures and synthetic pathway of N-oligosaccharides.

β1,6-Branched N-oligosaccharides are tri- and tetra-antennary complex-type N-glycans formed due to the action of N-acetylglucosaminyltransferase V (GnT-V), which catalyses the transfer of GlcNAc from UDP-GlcNAc to the 6-OH position of α-Man residue in α6 arm (**Figure 1**). In malignant transformation, the increased β1,6-branching is a result of enhanced activity of GnT-V associated with the increased expression of GnT-V gene (i.e. *Mgat5*), which is in turn regulated by Ras/Raf/MAPK, a signalling pathway commonly activated in tumour cells, and the Ets family including Ets-1 transcription factor [48, 49]. Ets-1 transcription factor, in turn, is known to regulate several molecules associated with cell invasiveness and metastasis, such as cyclin D (cell-cycle progression), vascular endothelial growth factor and basic fibroblast growth factor (potent angiogenic factors), Rho/Cdc42/rac-1 (motility) as well as matrix metalloproteinases-2, -3 and -9 (tissue remodelling) [50]. Artificial and spontaneous melanoma hybrids of high-metastatic potential have been proved to possess enhanced expression of GnT-V gene (*Mgat5*), increasing enzymatic activity of encoded glycosyltransferase [51]. Primary tumours are often infiltrated by macrophages and lymphocytes. The increased GnT-V activity and growing amount of β1,6-branched N-oligosaccharides in melanoma cells could reflect previous fusion of tumour-associated macrophages with cells of the primary tumour [52, 53]. Indeed, it has been shown that macrophage × Cloudman S91 mouse melanoma hybrids displayed increased motility *in vitro* and enhanced metastatic potential *in vivo* as well as up-regulated GnT-V activity and increased content of β1,6-branched N-glycans [51]. In macrophage-melanoma cell fusion hybrids β1,6-branched N-oligosaccharides have also been shown to be associated with enhanced melanin production and autophagy [54, 55]. A study using GnT-V knockout mice has demonstrated that although *Mgat5* products were not essential for embryonic development, when expressed in cancer cells they directly promoted tumour growth and metastasis [56].

β1,6-Branched N-oligosaccharides have been shown not to be synthesised by melanocytes or by cells of early melanoma *in situ*, but these structures are frequently found in a fully developed form of melanoma *in situ* as well as invasive and metastatic melanoma [55]. Our group, by a comparative analysis of glycoprotein pattern in four human cell line stages (primary site—WM35 cells; metastatic sites—WM9, WM239 and A375) with the use of lectins has revealed that melanoma cell lines from metastatic sites possessed more proteins being carriers of β1,6-branched N-oligosaccharides as well as α2,3- and α 2,6-linked sialic acids than those from melanoma *in situ*, as revealed by staining with *Phaseolus vulgaris* (PHAL), *Maackia amurensis* and *Sambucus nigra* agglutinins, respectively [57]. Not only the amount of β1,6-branched N-oligosaccharides progressively increased with disease progression, but also additional bands within the range of 100–160 kDa were observed by staining with PHAL. The minor differences in high-mannose-type glycan composition have also been observed in the above-mentioned four melanoma cell lines [57]. The functional importance of these type of oligosaccharides in tumourigenesis is still being studied; however, it has been shown that enhanced expression of high-mannose-type glycans on B16 murine melanoma cells promoted liver metastasis formation via mannose receptor-mediated melanoma cell attachment to hepatic sinusoidal endothelium [58]. Our further studies carried out on over 100 melanoma cell lines deposited in ESTDAB melanoma Cell Bank (Tubingen, Germany) have shown that the average number of proteins bearing β1,6-branched N-oligosaccharides was similar in uveal as well as primary

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and metastatic cutaneous melanoma cell lines [59]. Additionally, the expression of *Mgat5* was stated to be generally at low level; however, in 10% of cells its expression was high, while it was absent in only one cell line [59]. Comparative research on cancer-related N-glycan alteration in human melanoma WM793 cell line, which originated from early vertical growth phase lesions, and in its metastatic counterpart WM1205Lu cell line, from metastasis site in the mouse lung, has demonstrated that *Mgat-5* expression and the amount of β1,6-branched N-glycans increased with acquisition of a metastatic phenotype by melanoma cells [60]. In human melanoma biopsies, primary tumours showed heterogeneous staining for β1,6-branched N-glycans while metastases were much more homogeneous [61], suggesting that the presence of these glycans in primary tumours might be a sign of the increased metastatic competence.

It is well documented in the literature that elevated level of β1,6-branched N-oligosaccharides correlates with higher invasive potential, metastasis formation, reconstruction of the vascular system and growth of tumour cells [48]. Additionally, the loosened matrix adhesion of tumour cells may allow them to leave their original site in the tissue [62–64]. It is still a subject to identify glycoproteins bearing these structures. It is evident from the studies of our group that the expression level of β1,6-branched N-oligosaccharides is associated with acquisition of the metastatic potential in melanoma, and of particular interest are glycoproteins with the apparent molecular weight of 100–160 kDa [57]. We identified target glycoproteins of GnT-V from four human melanoma cell lines (WM35, WM9, WM239 and A375) by tandem mass spectrometry [48, 65]. Among the identified proteins, the largest group comprised integrin subunits (α2, α3, α4, α5, αv, β1 and β3). Additionally, N-cadherin, L1CAM, Mac-2 binding protein (Mac-2-BP), lysosome-associated membrane protein 1 (LAMP-1), CD44, melanoma-associated antigen (MAA), melanoma cell adhesion molecule (CD146, Mel-CAM), intracellular adhesion molecule 1 (CD54, ICAM-1), tumour rejection antigen-1 and melanoma-associated chondroitin sulphate proteoglycan 4 were found. The number of proteins being a substrate for GnT-V seemed to be better correlated with melanoma development and progression than with the expression of these cell adhesion molecules on melanoma cell surface. Independently of melanoma progression, αv and β1 integrin subunits as well as LAMP-1, CD146, CD54 and Mac-2-BP were always substrates for GnT-V; α3, α5 and β3 integrin subunits possessed no β1,6-branched N-oligosaccharides in WM35 cell line, being a radial growth phase primary melanoma, whereas α4 integrin subunit, CD44 and N-cadherin appeared to have these structures only in A375 cell line, which was the most aggressive melanoma cell line among the studied ones. It is well documented in the literature that the patterns of cell adhesion molecules differ between normal and malignant tissues. In cutaneous melanoma, the expression levels of α2β1, α3β1, α6β1 and αvβ3 integrins have been found to be associated with tumour progression. We demonstrated that not only gain or loss of adhesion molecule expression and increased level of β1,6-branched N-oligosaccharides, but also changes in the number of proteins being a substrate for GnT-V appear to be a consequence of disease progression from a tumourigenic to the metastatic phenotype. The involvement of these glycoproteins in adhesion and migration of cutaneous melanoma cells has been clearly demonstrated [60, 62, 63, 66–71]. In general, overexpression of β1,6-branched N-glycans on cell adhesion molecules contributed to the significant decrease in these cell adhesion level to extracellular matrix components, loss of contact inhibition as well as increased motility *in vitro* and enhanced

metastasis *in vivo*. Similarly, the presence of α2,3- and α2,6-linked sialic acids on the cell surface has facilitated the migratory properties and enhanced invasive properties of human melanoma cells, respectively [70]. The loosened matrix adhesion of tumour cells permits them to leave their original site in the tissue. This, in turn, may facilitate the turnover of cell-cell and cell-extra-cellular matrix contacts to enhance cell motility [17, 72].

Contrary results regarding the role of β1,6-branched N-glycans were obtained when glycosylation analysis was performed on tissues isolated from patients. Studies carried out on sections of 100 primary cutaneous malignant melanoma histochemically stained with five lectins (*Sambucus nigra* agglutinin, *Phaseolus vulgaris* agglutinin, *Triticum vulgaris* agglutinin, *Maackia amurensis* agglutinin and *Helix pomatia* agglutinin) differing in their carbohydrate binding specificity, have revealed that β1,6-branched N-glycans and sialic acid residues are of no functional importance in melanoma [73], although both of them were correlated with metastasis in other malignancies (breast, lung and colon). These results obtained by Thies and colleagues [73] clearly showed that only N-acetylgalactosylamine/-glusocamine residues, recognised by *Helix pomatia* agglutinin might be linked to metastasis in human malignant melanoma. The predominant expression of Thomsen-Friedenreich (T) antigen (Galβ1-3GalNAcα1-O-Ser/Thr) versus its immature precursor (GalNAcα1-O-Ser/Thr), both of which are an uncompleted form of O-linked mucine-type glycans, has been found to be helpful in order to differentiate primary melanomas from metastatic ones [73]. The close association between Tn and sialyl-Tn antigens and neoplastic transformation prompted some researches to use them for active immunotherapy [74]. Our group has shown that nucleolin, the expression of which is positively correlated with the increased rate of cell division, was a carrier of Tn antigens and was present on the cell surface of melanoma cells [75]. As this molecule contains multiple possible MHC class I binding peptides in its sequence, it might be a target for immunodiagnostic and possibly therapeutic purposes. Interestingly, it has been found that N-glycosylation enhanced presentation of a MHC class I-restricted epitope from tyrosinase [76]. This enzyme is a membrane-associated glycoprotein and acts as antigens for immunological recognition of melanomas [77].

It has also been shown that B16 melanoma cells exhibited a fivefold higher cell surface β1,4-galactosyltransferase activity in metastatic variants than their non-metastatic counterparts [78]. This enzyme catalyses the transfer of galactose residue to terminal N-acetylglucosamine residues on the cell surface glycolipid glucosylceramide (**Figure 2**), which is a precursor of glycosphingolipids. Glycosphingolipids which possess at least one sialic acid residue constitute a broad family of molecules called gangliosides. It has been shown that gangliosides are expressed with higher abundance in tumour cells in comparison to their normal counterparts. In normal melanocytes GM3 are expressed as their major gangliosides, whereas GM3 and GD3 are synthesised predominantly in malignant melanomas [79, 80]. A few melanomas frequently synthesise small amounts of more complex gangliosides, i.e. GM2 and GD2. In human melanomas the level of GD2 expression has been found to be associated with tumour progression and metastatic potential [81].

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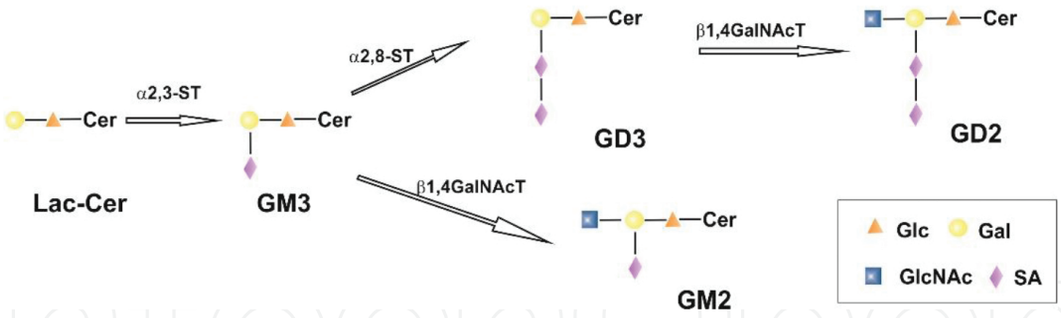

Figure 2. Structures and synthetic pathway of major gangliosides.

Ganglioside expression on individual cell lines is regulated by the availability of a precursor, the expression level of β1,4-N-acetylgalactosaminyltransferase, α2,8-sialyltransferase activity and the compartmentation of the glycosylation machinery of a cell [80, 82]. Total ganglioside level has been shown to be a potential tool for evaluating the response to immunotherapy in melanoma patients [83, 84]. Furthermore, GM3 and GD3 shed by tumour cell into the microenvironment enhance tumour formation and are able to promote severe immune dysfunction [85–87]. At present, a number of gangliosides are considered to be attractive targets for cancer immunotherapy. GM3 is the precursor of the ganglioside family members that contain either N-glycolylneuraminic acid (Neu5Gc) or N-acetylneuraminic acid (Neu5Ac). The presence of Neu5Gc is a result of the expression of cytidine monophospho-N-acetylneuraminic acid hydrolase [88]. Although Neu5Gc-containing gangliosides are not self-antigens in humans [89], they have been described as tumour antigens in several human cancers including melanoma, lung and breast cancer [90–92]. The presence of Neu5GcGM3 on the cell membrane has been shown to promote cell proliferation and adhesion *in vitro* as well as tumour growth *in vivo* [93, 94]. Thus, Neu5GcGM3 are regarded as attractive targets for cancer immunotherapy [95]. Indeed, NeuGc5GM3-based vaccine composed of very small size proteoliposomes (VSSP) resulting from the hydrophobic conjunction of GM3 gangliosides with *Neisseria meningitides* membrane protein used in mice bearing early-stage B16-F10 melanoma tumours induced a complete anti-tumour protection in all mice [86, 93, 94]. Moreover, 1E10 monoclonal antibody, a murine anti-idiotypic antibody that mimics Neu5GcGM3, has been tested in several clinical trials for melanoma, breast and lung cancer as an anti-idiotypic cancer vaccine [96]. Also GD3, the most abundant ganglioside, has been used as an immunogen for trial vaccination against malignant melanoma [97]. Moreover, 9-O-acetylation of sialic acid containing gangliosides, especially 9-O-Ac-GD3 in human melanoma cells, provides a unique antigenic determinant, which is absent in normal human melanocytes [98]. Additionally, O-acetylated sialic acid residues reduce susceptibility of tumour cells to degradation and extend their lifetime *in vivo*, as well as may stimulate cellular growth and suppress cellular differentiation [99].

One of the important basic features of melanoma cells is the property to change their adhesive interactions with other cells (keratinocytes, fibroblasts, endothelial and immune cells) and extracellular matrix protein. This property is a key element in the acquisition of the potential

to detach from their original site of tumour growth, invade surrounding tissues and, finally, metastasise. These interactions are mediated by cell adhesion molecules belonging to integrins, cadherins as well as immunoglobulin (Ig) superfamily. Changes in the expression and/or function of integrins, cadherins, CD44, Mel-CAM/MUC18 and intercellular adhesion molecule -1 have been documented in the progression of primary melanoma [100, 101]. It is also well documented in the literature that changes in cell adhesion molecules and growth factor receptors as a consequence of their oligosaccharide modification are associated with the function and biological behaviour of cancer cells. In the case of the cell surface receptors, the changed glycosylation pattern may affect their conformational stability, binding ability as well as their presence in the cell membrane. Details on changes in glycosylation patterns of cadherins and integrins that can modify the adhesive properties of melanoma cells are presented in two other chapters of this book.

The function of CD44, a widely distributed membrane glycoprotein belonging to the Ig superfamily, which abnormal expression on tumour cells enhances the ability to grow and metastasise *in vivo*, has been demonstrated to be partially influenced by O-linked oligosaccharides added to Ser/Thr-rich regions encoded by variable spliced CD44 exons [102]. In addition, all potential N-glycosylation sites in CD44 molecule have been proved to be necessary to maintain hyaluronic acid-recognition domain in the appropriate conformation [103]. Moreover, glycosylation of CD44 due to GnT-III enhanced B16-hm melanoma cell adhesion to hyaluronan, local tumour growth and metastatic growth in the spleen [104]. Other studies have shown that the presence of LAMP-1 with polylactosamine moieties on the cell surface of melanoma cells mediated organ-specific metastasis via lectin receptors on the lung vascular endothelium [105]. It has also been demonstrated that binding of a soluble ligand of SHPS-1 (i.e. CD47) to B16-F10 mouse melanoma cells was dependent on proper glycosylation of SHPS-1 molecule, another member of Ig superfamily, which plays an important role in integrin-mediated cytoskeleton reorganisation and migration, and that this defect renders melanoma cells resistant to CD47-induced inhibition of cell motility [106]. Interestingly, it has also been shown by deletion mutants that for P-glycoprotein function, which is a large and heavily glycosylated membrane protein conferring multi-drug resistance by pumping out a range of different drugs from the cell, N-glycosylation was necessary for its proper routing or stability but not for drug transport [107]. It is known that progression of melanoma correlates with the enhanced expression of glucose-regulated protein of 78 kDa (GRP78) and the increase in anti-GRP78 IgG serum titres in patients [108]. It has been shown that the glycosylation of anti-GRP-78 IgG changes as the disease progresses and the hyperglycosylated auto-antibodies stimulated cell proliferation via Akt signalling pathways [108].

It is well known that cancer metastases show organ selectivity and one of the important factors that determines the selectivity is the affinity of tumour cells towards the cells of an organ involved in metastasis. Most of the cell lines expressing β1,6-branched N-glycans have been shown to metastasise either to the liver or to the lung. β1,6-branched N-glycans are the preferred intermediate for the extension with polylactosamine chains (i.e. Galβ1,4GlcNAc β1,3- repeating units of 2 to more than 10 in length). Polylactosamine chains can be capped with various sequences, including Lewis antigens, sialic acids and fucose residues. It has been

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shown that lysosomal-associated membrane protein 1 (LAMP-1) present on the cell surface of high-metastatic murine B16-F10 cells was a carrier of a very high level of β1,6-branched N-glycans substituted with polylactosamine chains [105]. These structures were proved to be the key determinants in B16-F10 cells for preferred metastasising to the lungs, and organ-specific adhesion and metastasis was mediated via galectin-3 expressed in the highest amount on the lung vascular endothelium [105, 109, 110]. Complexes of galectin-3 with β1,6-branched N-glycans substituted with polylactosamine facilitated not only the initial arrest, but also took part in all the subsequent steps of extravasation and organ colonisation. Lung colonisation may also be realised by E-selectin-mediated interaction, but B16-F10 cells did not appear to use this molecule. B16-F10 cells transfected with cDNA encoding both α1,3- and α1,4-fucosyltransferases that catalyse reactions leading to the synthesis of sialyl-Lewis X and sialyl-Lewis A antigens, respectively, were not able to form metastasis in the liver in C57BL/6 mice, but formed numerous liver metastasis in E-selectin transgenic mice [111]. This means that sialyl-Lewis X (SAα2,3Galβ1,4(Fucα1,3)GlcNAcβ-) antigens on β1,6-branched N-glycans extended with polylactosamine chains did not serve as the ligand for lung colonisation in B16-F10 cells [105]. Human melanocytes did not express sialyl-Lewis X antigens and poorly expressed sialyl-Lewis A (SAα2,3Galβ1,3(Fucα1,4)GlcNAcβ-) antigens; however, these structures are overexpressed on cultured melanoma cells and melanoma tissue biopsies [112]. These findings indicated that sialyl-Lewis X and sialyl-Lewis A antigens are neoplastic differentiation antigens of human melanoma. Moreover, it has been proved that acquiring the expression level of sialyl-Lewis X antigens through the transfection of α1,3-fucosyltransferase III dramatically increased the metastatic capability of human melanoma MeWo and mouse melanoma B16 cells [113, 114]. However, T antigen, another potential ligand for galectin-3, has not been involved in mouse lung-specific metastasis [109]. Nevertheless, α 2,3-linked sialic acids on the surface of B16-F10 cells have been demonstrated to play an important role in lung metastasis [115]. The use of soyasaponin I, an inhibitor that reduces the expression of α 2,3-linked sialic acids, not only enhanced cell adhesion to extracellular matrix proteins, reduced cell migration, but also reduced the ability of melanoma cells for lung colonisation in mice. The positive correlation between the level of α1,6-fucosylated biantennary N-glycans on B16 murine cells and cellular potential to metastasise to the lung has been also demonstrated [116]. It has also been shown that cells expressing multi-antennary N-oligosaccharides un-substituted with polylactosamine chains home in the liver which expresses galectin-1 [117].

Recent advances in the discovery of microRNA (miRNA) role in cutaneous melanoma pathogenesis have revealed that miRNA can affect the cell surface proteins by interfering with their post-translational modifications. It has been found that overexpression of *miR-30b* and *miR-30d* in primary and metastatic tissues induced changes in glycosylation profiles of the membrane-bound proteins [118]. Moreover, *miR-30b* and *miR-30d* up-regulation was correlated with the stage, metastatic potential, shorter time to recurrence and reduced overall survival. *GALNT7* was found to be the specific gene target that mediated these pro-metastatic effects. GALNT7 belongs to GalNAc transferases, which initiate mucin-type O-linked glycosylation in the Golgi apparatus [119]. Another consequence of *miR-30b* and *miR-30d* overexpression was the reduction of CD3+ T cells recruitment and accumulation of regulatory T cells at the metastatic site *in vivo*, which could be partially mediated by increased secretion of IL-10. Thus,

targeting *miR-30b/30d* in melanoma cells could counteract both with its pro-metastatic and immunosuppressive effects by de-repressing GALNT7 endogenous level.

To date, one monoclonal antibody TM10 produced from mice vaccinated with FasL-expressing B16-F10 mouse melanoma cells was able to recognise a range of human tumour cell lines, including melanoma [120]. Despite the fact that over 50% of cancers express tumour-associated carbohydrate antigens, such as gangliosides, Lewis antigens and Thomsen-Friedenreich antigen, none of them was the antigenic target of TM10. However, another monoclonal antibody HMB45 that recognises melanoma-specific target, i.e. Pmel17/gp100, reacts with its sialylated RPT domain [121].

4. Glycation and melanoma

The risk of melanoma is significantly associated with high fasting glucose, which is entirely independent on age, body mass index and smoking [122]. A non-enzymatic reaction (glycation) between ketones or aldehydes and amino groups of proteins contributes to the ageing of proteins and leads to the formation and accumulation of irreversible cross-linked protein derivatives termed advanced glycation end products (AGE). AGE have been proved to stimulate growth and migration of malignant melanoma *in vitro* and *in vivo* through the interaction with the receptor for advanced glycation end products (RAGE) [123–126]. RAGE is known to stimulate multiple signalling pathways crucial for cell migration, such as p38 mitogen-activated protein kinase, Ras-extracellular signal-regulated kinase 1/2, stress-activated protein kinase/c-Jun-NH2-terminal kinase and Cdc42/Rac pathways. Recently, RAGE expressed on mouse melanoma B16 cells has been identified as a crucial factor for pulmonary metastases of these tumour cells [127]. Interestingly, RAGE has also been identified as a potential anti-metastatic drug target [128].

RAGE has been shown to interact with S100B [129], which is a serological biomarker widely used in clinical practice to determine the prognosis for patients with distal melanoma metastases, despite the fact that it fails to detect melanoma progression in up to 20% of patients [130–133]. Recently, it has been demonstrated that among stage III–IV melanoma patients, decreased serum levels of soluble forms of RAGE (sRAGE and esRAGE) correlated with poor overall survival [134]. Interestingly, it has also been proved that overexpression of RAGE in WM115 human primary melanoma cells was associated with mesenchymal-like morphology of the cell, a switch to a metastatic phenotype as well as up-regulation of S100B [126]. As the elevated level of S100B is known to down-regulate p53 suppressor protein, small-molecule inhibitors targeting S100B-p53 interaction are currently under intense investigations as new therapeutic strategies for malignant melanoma [135, 136]. It has also been shown that the expression of S100P, which is another member of the S100 family, was significantly higher in malignant melanoma than in primary melanoma [137]. Abnormal level of S100P can contribute to tumour growth, invasion and metastasis [138]. Other functional ligands for RAGE include chondroitin sulphate containing E disaccharide units (GlcNAβ1-3GalNAc(4,6-O-disulfate)β1-) and heparin sulphate [127].

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5. Conclusions

Alterations in glycans on proteins and lipids have long been associated with malignant transformation. The observed modifications are either a direct consequence of the oncogenic process or an indirect effect of changes occurring in the tissue environment and inflammation. Analytical efforts in melanoma glycomics contribute to understanding the role of cellular and molecular properties of cells that influence the dissemination of tumour cells, which might be essential for understanding the pathogenesis of tumour development and metastasis. These studies also offer considerable possibilities for screening, selection and identification of differentially expressed glycoconjugates, in order to develop non-invasive, sensitive and discriminative *in vitro* diagnostics tests. Unfortunately, the vast majority of studies have been performed on melanoma cell lines and mouse model systems due to the insufficient number of samples obtained from melanoma patients. Therefore, there is still limited evidence on whether the observations made in these models are consistent with the role of glycosylation in tumour tissues. Further studies based on human tissues are needed to establish functional impact of glycosylation changes on human melanoma as well as detection and discovery of glycan motifs in melanoma samples similarly to advances achieved in lung, liver, colorectal, brain, prostate, breast and ovarian cancer researches.

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